How close are we to implementing gene targeting in animals other than the mouse?

Mario R. Capecchi*
Howard Hughes Medical Institute, University of Utah School of Medicine, Salt Lake City, UT 84112-5331

The paper by Kubota *et al*. in this issue of PNAS (1) describes several significant contributions that bring us much closer to extending ''gene targeting'' to mammalian species other than the mouse. Gene targeting now provides the means for creating new strains of mice with mutations in virtually any gene. First, the desired mutation is introduced into a cloned copy of the chosen gene by standard recombinant DNA technology. The mutation then is transferred to the genome of a pluripotent mouse embryo-derived stem (ES) cell by means of homologous recombination between the exogenous mutated DNA sequence and the cognate DNA sequence in the ES cell chromosome. By microinjection of ES cells containing the targeted mutation into blastocysts and allowing the embryo to come to term in foster mothers, chimeric mice can be generated that are capable of transmitting the targeted mutation to their offspring.

The power of gene targeting is that the investigator chooses both which genes to modify and how to modify them. The investigator has virtually complete control over the way in which the gene's DNA sequence or surrounding regulatory elements are modified. Thus, gene targeting should not be thought of as a means only for the inactivation of genes, but as a method for altering gene activity in any purposeful manner. This technology permits evaluation of deliberately selected gene functions in the intact mouse and a systematic dissection of the most complex of biological processes, such as development and learning. Because nearly all biological phenomena are mediated or influenced by genes, this technology has had a profound impact on all areas of biological research in mammals, including the study of cancer, immunology, neurobiology, and human genetic disease.

The extension of gene targeting to other mammalian species would provide an additional boost to the study of mammalian biology. To date, however, efforts to isolate embryo-derived stem cells capable of contributing to the formation of the germ line in mammalian species, other than the mouse, have failed. This failure precludes the use of such cells to generate chimeras of other mammalian species, and thus use of the gene targeting strategy that has been so successful in the mouse. However, Wilmut's successful cloning of the sheep, Dolly, by nuclear transfer of a somatic cell nucleus into an enucleated sheep oocyte (2) provides an alternative route to generating animals with targeted mutations in their germ line. Targeted mutations can be introduced readily into any somatic cell in culture. (In fact, our experience suggests that if the experiments are done appropriately, using isogenic DNA, optimizing the tissue culture selection procedures for the chosen cell line, etc., most mammalian cells in culture show closely equivalent capacities to mediate homologous recombination between exogenous and endogenous DNA sequences.) The transfer of nuclei from cultured somatic cells containing targeted mutations to enucleated oocytes could provide the desired alternative route. The potential for success of such an approach is supported by the experiments reported by Kubota *et al*. (1). Those authors have generated cloned animals of a 17-year-old bull by using nuclei of fibroblasts cultured from a biopsy of his skin. Culture of these fibroblasts *in vitr*o for 10–15 passages ($\approx$ 20–30 cell doublings) did not compromise their capacity to generate healthy animal clones.

These new results have made two significant contributions to the prospects for use of this procedure as the means of generating animal clones containing targeted gene modifications. First, the authors used the most easily obtainable cells as a source of transplanted nuclei. Under the commonly used culture conditions for tissue explants, the cell type that generally outgrows all others is the fibroblast. The selection of fibroblasts as a source of nuclei for producing animal clones makes this procedure particularly practical. Second, as demonstrated by Kubota *e*t *al*. (1), these fibroblasts can be cultured *i*n *vitr*o for 10–15 passages without losing their capacity to generate healthy bull clones is very significant because this number of passages exceeds the number needed to generate purified populations of cells containing targeted mutations.

Although all mammalian somatic cells have the capacity to mediate homologous recombination (a requirement to generate targeted mutations), the efficiency of this reaction is low relative to the competing nonhomologous recombination reaction (3). Overcoming this deficit requires the use of tissue culture selection procedures to enrich for cells containing the desired homologous recombination event (4). However, because even under the most extreme conditions the absolute targeting frequency is approximately one in 10^5-10^6 starting cells (i.e., fewer than 20 cell doublings), the ability to culture the donor fibroblasts for at least 30 cell doublings, while still retaining their animal cloning potential, provides an ample cushion to carry out sophisticated gene targeting procedures with these cells. This procedure could include even the possibilities of generating cells homozygous for the targeted modification, or ones containing conditional mutations (5).

A problem associated with all animal cloning procedures reported to date is high rates of embryonic and neonatal lethality. The source of this lethality has not been established. However, a possible culprit is improper execution of the complex changes in the patterns of genomic demethylation and methylation that normally accompany the process of early embryogenesis. Proper execution of this program is required to maintain balanced growth between extraembryonic and fetal tissues (6). This process normally is accomplished by differential epigenetic imprinting of selected genes from the maternal and paternal genomes responsible for control of balanced growth. A cloning procedure, based on the introduction of somatic nuclei into the cytoplasmic environment of the oocyte, requires a reprogramming of the somatic methylation pattern such that it now can simulate the complex dynamic methylation and de-methylation patterns that take place during normal embryonic development. To my knowledge prior successful cloning experiments have used only nuclei from female donors. Thus, it is also significant that the donor used by Kubota *e*t *al*. (1) as a source of their nuclei was not only an old animal (i.e., 17 years old), but also a bull. This experiment unequivocally demonstrates that both male and female somatic nuclei can be reprogrammed successfully to drive normal embryogenesis. Before these experiments, researchers entertained the possibility that only female somatic cells retain the capacity to be properly reprogrammed by an oocyte environment. The presumed failure was attributed to incompatibilities between male somatic nuclei and female oocyte cytoplasm for attaining the appropriate de-methylation/methylation states required to support successful embryogenesis.

Though there are obvious commercial incentives pushing animal cloning in domestic species, such as cattle, the mouse appears to be a more suitable animal for such experimentation (7). It therefore seems ironic that the frequency of reported success in animal cloning appears to be higher in the larger animals than in the mouse. Although it may be dangerous to make comparisons of efficiencies across such disparate species, involving different experimenters and differing protocols, the growing inequality of reported success invites comment. One factor that might contribute to a difference in cloning efficiency is a possible timing difference associated with the very early cell divisions as to how rapidly the zygotic gene products are required to sustain normal development in various species. Differences in these parameters could contribute to the amount of time a somatic nucleus, on entering the oocyte cytoplasmic environment, has to change its inherent somatic program to the embryonic program. This line of reasoning suggests that artificial prolongation of these early cellular events could provide a means of increasing the efficiency of derived mouse clones.

A reasonable question from the public is whether such animal cloning experiments should be supported. Cloning inevitably will be used to increase the commercial value of livestock. Domestic animals, as well as plants, are being examined for their potential to produce pharmaceuticals. One readily identifiable, and likely popular, future use of this technology is conservation of endangered species. There is no reason to suspect that fibroblasts from endangered species could not be frozen so that at a later time, their nuclei might serve as donors to generate animal clones of that species. Archiving of such cells, at a time when sufficient genetic

diversity of the endangered species still exists, could reduce potential future risks associated with attempts to maintain these species when genetic diversity has declined.

There are also numerous human medical problems that might be solved through the combined use of gene targeting and animal cloning. One prominent one can be offered as an example. Currently, the need for organs suitable for human transplantation far exceeds their supply. This problem had led to the suggestion that organs from a domestic animal, such as a pig, might be used in place of human organs. Such approaches are not presently feasible because of the rapidity with which pig tissue would be rejected by the human immune system. However, it is conceivable that gene targeting and cloning could be used to alter the molecules in pig tissue that trigger immunological intolerance, thereby producing usable pig donors of transplantable organs.

An additional concern from the public is that, as the success of animal cloning increases, so does the probability that this technology will be used to generate human clones. This may be true, particularly in the private sector that is not as easily regulated as are, for example, academic or medical institutions. Personally, I would not be particularly concerned if a very wealthy, eccentric individual desired to produce a clone of him or herself. If the intent were to reproduce an exact copy, the wealthy cloner very likely would be disappointed with the results. We have lived with genetic clones, identical twins, for as long as the human race has existed. We have survived with these clones, and we readily recognize their individuality. To the extent that environment contributes to individuality, a cloned offspring is likely to be more different from its parent than are identical twins reared in a similar environment from each other. The further the upbringing of the cloned offspring is separated in time and surroundings from the rearing of its parent, the lower the probability of similarities in their environmental histories. Would the clone be subject to health risks not experienced by the parent? Possibly, data that would allow us to evaluate such potential risks as reduced longevity, or the effects resulting from accumulation of somatic mutations, are not yet available from animal cloning experiments. For all of these reasons, cloning of humans should not be a very attractive option for the sane, even if wealthy and eccentric, individual.

However, human cloning could provide an option to couples unable to have genetically related children by any other means. The biological drive that supports the desire to have one's own genetically related children is extremely strong. Under these circumstances, the risks to the offspring would have to be very carefully evaluated and explained to prospective parents. However, denying parents the right to have their own genetically related children also raises enormous ethical issues. Most of us would have great difficulties in being participants to such decisions. With the development of new technologies such as cloning come the responsibilities for social deliberations that seek ways to use this technology in productive ways that keep the interests of individuals and society in balance.

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